



The Role of Nuclear Factor-E2-Related Factor 1 in the Oxidative Stress Response in MC3T3-E1 Osteoblastic Cells

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Background: Reactive oxygen species (ROS) and antioxidants are associated with maintenance of cellular function and metabolism. Nuclear factor-E2-related factor 1 (NFE2L1, Nrf1) is known to regulate the expression of a number of genes involved in oxidative stress and inflammation. The purpose of this study was to examine the effects of NFE2L1 on the response to oxidative stress in osteoblastic MC3T3-E1 cells.

Methods: The murine calvaria-derived MC3T3-E1 cell line was exposed to lipopolysaccharide (LPS) for oxidative stress induction. NFE2L1 effects were evaluated using small interfering RNA (siRNA) for *NFE2L1* mRNA. ROS generation and the levels of known antioxidant enzyme genes were assayed.

Results: *NFE2L1* expression was significantly increased 2.4-fold compared to the control group at 10 µg/mL LPS in MC3T3-E1 cells ($P<0.05$). LPS increased formation of intracellular ROS in MC3T3-E1 cells. *NFE2L1* knockdown led to an additional increase of ROS (20%) in the group transfected with *NFE2L1* siRNA compared with the control group under LPS stimulation ($P<0.05$). RNA interference of *NFE2L1* suppressed the expression of antioxidant genes including metallothionein 2, glutamate-cysteine ligase catalytic subunit, and glutathione peroxidase 1 in LPS-treated MC3T3-E1 cells.

Conclusion: Our results suggest that NFE2L1 may have a distinct role in the regulation of antioxidant enzymes under inflammation-induced oxidative stress in MC3T3-E1 osteoblastic cells.

Keywords: NF-E2-related factor 1; Oxidative stress; Osteoblasts

INTRODUCTION

Reactive oxygen species (ROS) are considered to be a causal factor in inflammation, aging and a number of degenerative diseases such as atherosclerosis, carcinogenesis, infarction, and

osteoporosis [1]. The effects of ROS are eliminated by enzymatic mechanisms involved in cellular antioxidant defense and xenobiotic detoxification [2]. The delicate balance between ROS and antioxidants is important to maintain equilibrium between osteoblasts and osteoclasts activities, respectively, under

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physiological conditions [3]. ROS and antioxidants are also known to be involved in the pathogenesis of bone loss such as in osteoporosis [4,5].

Lipopolysaccharide (LPS) is a constituent of the cell wall outer membrane of gram-negative bacteria and has various biological effects including immune and inflammatory responses [6]. LPS has the capacity to induce bone resorption *in vitro* and also stimulates osteoblasts to secrete osteolytic factors [7]. LPS is involved in the suppression of bone sialoprotein, a mineralized tissue-specific protein in osteoblast-like ROS 17/2.8 cells [8].

Nuclear factor-E2-related factor 1 (NFE2L1, Nrf1) is a basic leucine zipper protein (bZIP) in the Cap-N-Collar (CNC) transcriptional factor family and controls antioxidant response element (ARE)-driven genes [9]. NFE2L1 was originally suggested to have a role in β -globin gene expression in erythroid cells; however, NFE2L1 has also been shown to bind the ARE and regulate the expression of many genes involved in oxidative stress, cellular differentiation, and inflammation [10].

NFE2L1 can protect cells from oxidative stress by regulating genes encoding enzymes related to glutathione (GSH) biosynthesis and other oxidative defense enzymes [10]. Synthesis of GSH, a major antioxidant in the cell, involves γ -glutamylcysteine ligase (γ -GCL), which consists of a catalytic (GCLC) and a modifier (GCLM) light chain [11]. Evidence suggests that NFE2L1 regulates transcription of GSH synthesis-related genes and other antioxidant genes including NAD(P)H dehydrogenase, quinone 1 (NQO1), ferritin-H, metallothionein (MT)-1 and -2 in fibroblasts, and hepatocytes [12-15]. However, despite these observations, there have been no investigations of the function of NFE2L1 in oxidative stress and the NFE2L1-related antioxidant system in osteoblasts.

In the present study, we attempted to assess the effects of inflammation-induced oxidative stress on NFE2L1 expression pattern and also determined a role of NFE2L1 in the expressions of antioxidant-related enzymes using MC3T3-E1 osteoblastic cells.

METHODS

Cell culture and treatment

The murine calvaria-derived MC3T3-E1 osteoblast-like cell line was used in this study. MC3T3-E1 cells were maintained in α -modified minimum essential medium (α -MEM) containing antibiotics and 10% fetal bovine serum. This basic medium was replenished every 3 days.

MC3T3-E1 cells were seeded in either 96- or 6-well plates.

Cells were then treated with different concentrations of LPS (Sigma-Aldrich, St. Louis, MO, USA). Cells were subsequently washed twice with phosphate-buffered saline (PBS), and then cells were harvested for experiments.

Transfection of small interfering RNA

MC3T3-E1 cells were plated in either 96- or 6-well plates. After overnight culture, the cells were transfected using Lipofectamine PLUS reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Each transfection assay was performed with control small interfering RNA (siRNA) or *NFE2L1* siRNA (siNFE2L1) (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

RNA isolation and quantitative real-time polymerase chain reaction

Cultured cells were superficially washed with PBS, followed by extraction of total RNA using Trizol (Invitrogen) according to the manufacturer's standard instructions. Samples (2 μ g) of total RNA were reverse transcribed, followed by oligo (dT) primer and MMLV Reverse Transcriptase (Promega Co., Madison, WI, USA) at a final volume of 25 μ L. Aliquots of 2 μ L cDNA were used as templates for real-time polymerase chain reaction (PCR). PCR amplification was performed with 2 \times SYBR Premix Ex Ta (Takara Bio Inc., Shiga, Japan) and 10 pmol forward and reverse primers using Thermal Cycler DICE Real Time System (Takara Bio Inc.). Reactions were performed for 45 cycles of 95°C for 10 seconds, 60°C for 15 seconds, and 72°C for 30 seconds. Primers are listed in Table 1.

Measurement of intracellular ROS

Generation of intracellular ROS was measured according to the method described by Wang and Lou [16]. Briefly, MC3T3-E1 cells were cultured on 96-well plates (1×10^3 cells/well) and transfected with control siRNA (siCONT) or siNFE2L1. After 24 hours, cells were incubated in α -MEM containing fluorescent dye 50 μ M H2DCF-DA (Invitrogen) for 15 minutes in the dark, washed thoroughly by PBS, and further incubated in α -MEM with or without 10 μ g/mL LPS. The emitted fluorescence was measured by fluorometer (Wallac 1420D Fluorometer, PerkinElmer Inc., Turku, Finland) with excitation and emission wavelengths at 485 and 535 nm, respectively.

Statistical analysis

Statistical analysis was performed with SPSS version 11.5 (SPSS Inc., Chicago, IL, USA). Results are expressed as the

Table 1. Primers Used

Gene	Forward (5'-3')	Reverse (5'-3')
<i>β-Actin</i>	CCGCGAGCACAGCTTCTT	CCCACGATGGAGGGGAATAC
<i>NFE2L1</i>	GGAGAGCTTCCCTGCACAGT	TTACTTCCATAGCCTGCATTTC
<i>MT1</i>	ATGGACCCCAACTGCTCCT	ACAGCCCTGGGCACATT
<i>MT2</i>	CCGATCTCTCGTCGATCTCAACC	CAGGAGCAGCAGCTTTTCTTGACG
<i>GCLC</i>	GCACGGCATCCTCCAGTTCTT	TCGGATGGTTGGGGTTTGTC
<i>GCLM</i>	GGCTTCGCCTCCGATTGAAGA	TCACACAGCAGGAGGCCAGGT
<i>NQO1</i>	GCATTGGCCACACTCCACCAG	ATGGCCCACAGAGAGGCCAAA
<i>GPx1</i>	TGCTCATTGAGAATGTCGCGTCTC	AGGCATTCCGCAGGAAGGTAAAGA

NFE2L1, nuclear factor-E2-related factor 1; MT1, metallothionein 1; MT2, metallothionein 2; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; NQO1, NAD(P)H dehydrogenase, quinone 1; GPx1, glutathione peroxidase 1.

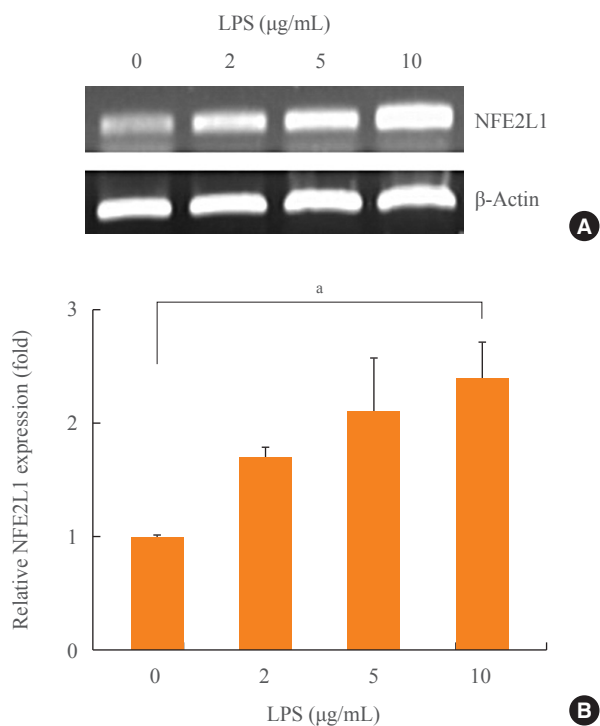


Fig. 1. The effect of lipopolysaccharide (LPS) on nuclear factor-E2-related factor 1 (*NFE2L1*) mRNA expression in MC3T3-E1 cells. Cells were treated with 0, 2, 5, or 10 μg/mL LPS for 24 hours. Levels of mRNA were analyzed by (A) semi-quantitative polymerase chain reaction (PCR) and (B) quantitative real time-PCR. The expression level of each mRNA was normalized to the *β-actin* levels. ^a $P < 0.05$ compared with the control group.

mean \pm SE and the statistical significance was determined by Student *t* test or one-way analysis of variance with Tukey *post hoc* test. Significance was defined by a $P < 0.05$.

RESULTS

NFE2L1 expression after LPS treatment

We examined the effect of LPS treatment on *NFE2L1* mRNA expression in MC3T3-E1 cells (Fig. 1). Cells were treated with 0, 2, 5, or 10 μg/mL LPS for 24 hours. Although LPS treatment showed a trend to stimulate the expression of *NFE2L1* gene in MC3T3-E1 cells dose-dependently, *NFE2L1* expression was significantly increased 2.4-fold compared to the control group at 10 μg/mL LPS ($P < 0.05$) (Fig. 1B).

RNA interference of *NFE2L1*

To investigate the contribution of *NFE2L1* to the oxidative stress response in MC3T3-E1 cells, specific siRNA for *NFE2L1* was transfected in MC3T3-E1 cells. Transfection with siNFE2L1 knocked down expression of *NFE2L1* by 79% at 20 nm siRNA as determined by quantitative real time-PCR ($P < 0.05$) (Fig. 2).

Effects of *NFE2L1* knockdown on ROS formation in MC3T3-E1 cells

MC3T3-E1 cells transfected with siCONT or siNFE2L1 were treated with LPS (10 μg/ml) for 10 minutes, and ROS generation was subsequently analyzed (Fig. 3). Quiescent cells (without stimulation) displayed a similar level of ROS in both siCONT and siNFE2L1 groups. LPS exposure resulted in a significant increase in the amount of ROS by 26% compared with unstimulated cells ($P < 0.05$). The siNFE2L1 led to an additional increase of ROS (20%) compared with the siCONT group under LPS stimulation ($P < 0.05$).

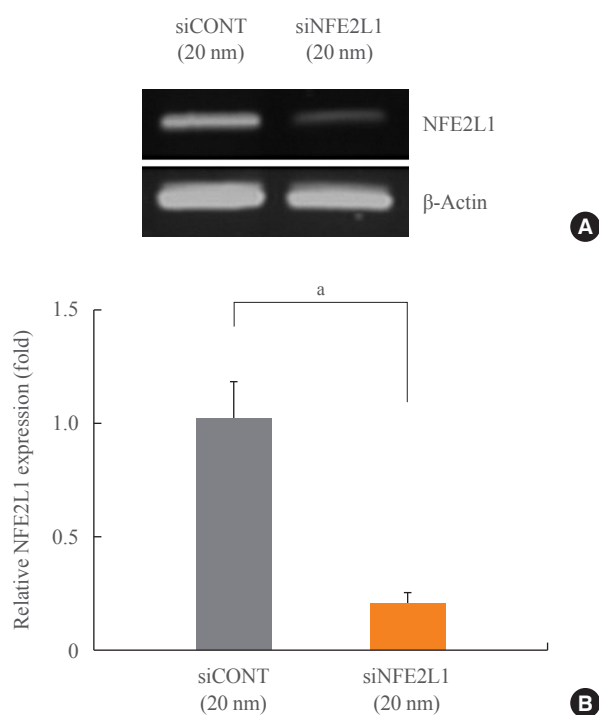


Fig. 2. Nuclear factor-E2-related factor 1 (*NFE2L1*) mRNA expression after transient transfection with control siRNA (siCONT) or *NFE2L1* siRNA (siNFE2L1) in MC3T3-E1 cells. Levels of mRNA were analyzed by semi-quantitative (A) polymerase chain reaction (PCR) and (B) quantitative real time-PCR. The expression level of each mRNA was normalized to the β -actin levels. ^a $P < 0.05$ compared with the siCONT group.

Effects of *NFE2L1* knockdown on antioxidant gene expression in LPS treated cells

We next examined the effect of mRNA inhibition of *NFE2L1* on the mRNA expression of antioxidant genes against oxidative stress in MC3T3-E1 cells. As shown in Fig. 4, there was no significant difference in antioxidant gene expression between siCONT and siNFE2L1 groups under unstimulated conditions. Exposure of MC3T3-E1 cells to LPS led to a significant increase in the level of *MT2* compared to untreated controls, and *NFE2L1* knockdown resulted in a decrease of 48% in *MT2* expression under LPS stimulation ($P < 0.05$) (Fig. 4B). In the presence of LPS, siNFE2L1 also significantly suppressed the expression of *GCLC* and glutathione peroxidase 1 (*GPx1*) by 41% and 37%, respectively, compared to the siCONT group ($P < 0.05$) (Fig. 4C, F). However, the differences in mRNA levels of *MT1*, *GCLM*, and *NQO1* between siCONT and siNFE2L1 groups following stimulation with LPS were not significant.

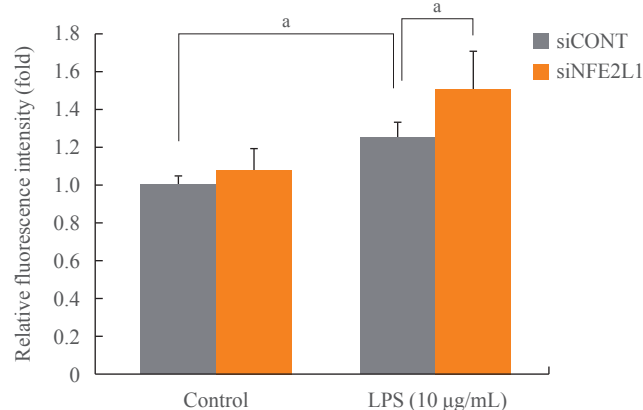


Fig. 3. Measurement of reactive oxygen species (ROS) with H2DCF-DA in MC3T3-E1 cells. Intracellular ROS in the transfectants of control siRNA (siCONT) and nuclear factor-E2-related factor 1 siRNA (siNFE2L1) were compared under control (no stimulation) and stimulations by lipopolysaccharide (LPS, 10 μg/mL) for 10 minutes. ^a $P < 0.05$ compared with the control group or siCONT cells.

DISCUSSION

Bone remodeling is controlled by a wide range of systemic factors including hormones and steroids and local factors as well as bacterial products such as LPSs. Bacteria-induced pathological bone remodeling is related to bacterial arthritis, osteitis, osteomyelitis, and periodontitis [7]. Bone loss at sites of infection results from mainly increased formation and function of bone-resorbing osteoclasts, though bacterially stimulated osteoblasts showed the ability to produce considerable inflammatory mediators that can promote osteoclastogenesis [17].

NFE2L1 is a member of the CNC family of bZIP transcriptional factors and plays an important role in the control of a wide range of genes involved in antioxidants, differentiation, and inflammation [10]. While an understanding of the role of *NFE2L1* in the stress response has been demonstrated in various cells, it remains unclear whether *NFE2L1* has unique functions under different conditions.

Here we found that expression of *NFE2L1* was increased dose-dependently by LPS in MC3T3-E1 cells. LPS is known to have biologic effects to stimulate the production of cytokines such as interleukin 1 (IL-1), IL-6, and tumor necrosis factor α from osteoblasts [7] and these factors contribute to produce ROS in nonphagocytic cells [18]. We then observed that ROS generation by LPS was additionally increased after *NFE2L1* silencing. Other studies have reported that *NFE2L1*-deficient hepatocytes and fibroblasts showed elevated free radicals under

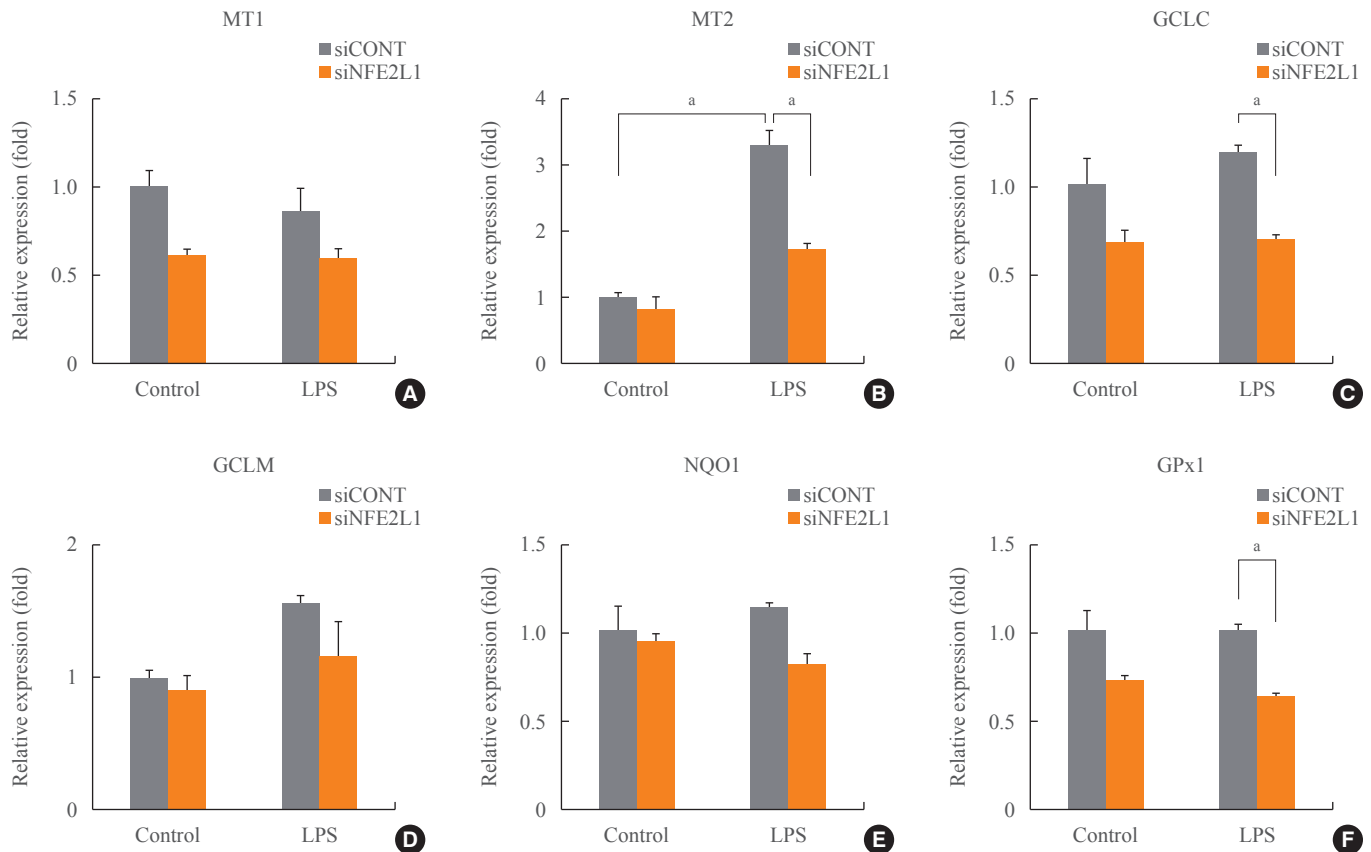


Fig. 4. The effect of nuclear factor-E2-related factor 1 (*NFE2L1*) knockdown on antioxidant gene mRNA expression in lipopolysaccharide (LPS) treated cells. Antioxidant genes are as follows: (A) metallothionein 1 (*MT1*), (B) metallothionein 2 (*MT2*), (C) glutamate-cysteine ligase catalytic subunit (*GCLC*), (D) glutamate-cysteine ligase modifier subunit (*GCLM*), (E) NAD(P)H dehydrogenase, quinone 1 (*NQO1*), and (F) glutathione peroxidase 1 (*GPx1*). MC3T3-E1 cells were transfected with control siRNA (siCONT) or *NFE2L1* siRNA (siNFE2L1) followed by 24-hour treatment of 10 μ g/mL LPS. Controls received culture medium only. Quantitation of mRNA levels was analyzed by quantitative real-time polymerase chain reaction. The expression level of each mRNA was normalized to the β -actin levels. ^a $P < 0.05$ compared with the control group or siCONT cells.

normal conditions and with cytotoxic agents, respectively [12,19]. Our results suggest that osteoblasts could deal with the increased intracellular oxidative stress burden partially mediated by NFE2L1.

In this study, LPS treatment led to a strong induction of *MT2* expression in MC3T3-E1 cells and functional inhibition of *NFE2L1* by siRNA caused a significant decrease in expression of *MT2* in the presence of LPS. The *MT* gene is known to be regulated transcriptionally *in vivo* by heavy metals, glucocorticoid hormones, and LPS [20-22]. The level of *MT2* expression is relatively higher than *MT1*, with the ratio of *MT1* mRNA to *MT2* mRNA ranging from 2:3 to 5:7 [23]. The mouse *MT1* and *MT2* genes are located in close proximity on chromosome 8 and are amplified together by heavy metals such as cadmium [23,24]. Although the ARE of the mouse *MT1* gene is preferen-

tially regulated by NFE2L1 [15], the presence of two forms of *MT* genes could allow greater flexibility in the regulation of expression depending on the different types of inducers. Therefore, our study suggests that LPS might act as a strong inducer of *MT2* expression in osteoblastic MC3T3-E1 cells, which is dominantly affected by NFE2L1 under oxidative stress.

We also observed that the expression of *GCLC* and *GPx1* was affected after NFE2L1 knockdown under LPS stimulation. The mRNA expression of *GPx1* and *GCLC* genes might be up-regulated to a negligible extent with LPS-only treatment. Li et al. [25] reported that the levels of GPx were not changed in LPS-stimulated macrophages but significantly elevated in LPS-activated cells exposed to an antioxidative stress reagent. Previous studies have shown that *GCLC* and *GPx1* were induced in response to overexpression of NFE2L1 or increased intracel-

lular accumulation of NFE2L1 [14,26]. The expression of *GCLM* and *NQO1* was not changed by LPS and knockdown of *NFE2L1*, as NFE2L2 has been shown to primarily regulate *GCLM* and *NQO1* [27,28].

Our study has several limitations. First, oxidative stress could be induced by many agents such as H₂O₂, high glucose, cytotoxic drugs and metal; however, we focused on inflammation-induced oxidative stress using LPS. Second, we did not assess the association of NFE2L1 with forkhead homeobox type O-1, which is a crucial regulator of oxidative stress in osteoblasts [29]. Third, NFE2L1 is known to involve in cellular differentiation [30,31]. It remains to be examined whether osteoblasts differentiation is affected by the change of NFE2L1 expression and antioxidant enzymes under LPS-induced oxidative stress. Finally, we only included the knock-down system of target gene, not performing over-expression system in this study.

In conclusion, this is the first study to elucidate the effects of NFE2L1 on inflammation-induced oxidative stress and the response of antioxidant enzymes in MC3T3-E1 cells. Our results show that the *NFE2L1* gene is induced by LPS treatment and NFE2L1 mediates expression of antioxidant enzymes under oxidative stress induced by LPS in osteoblastic MC3T3-E1 cells. This work suggests that NFE2L1 has a distinct function in regulating the response to oxidative stress in osteoblastic cells.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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REFERENCES

- Banfi G, Iorio EL, Corsi MM. Oxidative stress, free radicals and bone remodeling. *Clin Chem Lab Med* 2008;46: 1550-5.
- Lyakhovich VV, Vavilin VA, Zenkov NK, Menshchikova EB. Active defense under oxidative stress. The antioxidant responsive element. *Biochemistry (Mosc)* 2006;71:962-74.
- Sheweita SA, Khoshhal KI. Calcium metabolism and oxidative stress in bone fractures: role of antioxidants. *Curr Drug Metab* 2007;8:519-25.
- Maggio D, Barabani M, Pierandrei M, Polidori MC, Catani M, Mecocci P, et al. Marked decrease in plasma antioxidants in aged osteoporotic women: results of a cross-sectional study. *J Clin Endocrinol Metab* 2003;88:1523-7.
- Yalin S, Bagis S, Polat G, Dogruer N, Cenk Aksit S, Hattungil R, et al. Is there a role of free oxygen radicals in primary male osteoporosis? *Clin Exp Rheumatol* 2005;23: 689-92.
- Schletter J, Heine H, Ulmer AJ, Rietschel ET. Molecular mechanisms of endotoxin activity. *Arch Microbiol* 1995; 164:383-9.
- Nair SP, Meghji S, Wilson M, Reddi K, White P, Henderson B. Bacterially induced bone destruction: mechanisms and misconceptions. *Infect Immun* 1996;64:2371-80.
- Kato N, Nakayama Y, Nakajima Y, Samoto H, Saito R, Yamanouchi F, et al. Regulation of bone sialoprotein (BSP) gene transcription by lipopolysaccharide. *J Cell Biochem* 2006;97:368-79.
- Chan JY, Han XL, Kan YW. Cloning of Nrf1, an NF-E2-related transcription factor, by genetic selection in yeast. *Proc Natl Acad Sci U S A* 1993;90:11371-5.
- Biswas M, Chan JY. Role of Nrf1 in antioxidant response element-mediated gene expression and beyond. *Toxicol Appl Pharmacol* 2010;244:16-20.
- Lu SC, Ge JL, Kuhlenkamp J, Kaplowitz N. Insulin and glucocorticoid dependence of hepatic gamma-glutamylcysteine synthetase and glutathione synthesis in the rat. Studies in cultured hepatocytes and in vivo. *J Clin Invest* 1992;90: 524-32.
- Kwong M, Kan YW, Chan JY. The CNC basic leucine zipper factor, Nrf1, is essential for cell survival in response to oxidative stress-inducing agents. Role for Nrf1 in gamma-gcs(l) and gss expression in mouse fibroblasts. *J Biol Chem* 1999;274:37491-8.
- Lee TD, Yang H, Whang J, Lu SC. Cloning and characterization of the human glutathione synthetase 5'-flanking region. *Biochem J* 2005;390(Pt 2):521-8.
- Myhrstad MC, Husberg C, Murphy P, Nordstrom O, Blomhoff R, Moskaug JO, et al. TCF11/Nrf1 overexpression increases the intracellular glutathione level and can transactivate the gamma-glutamylcysteine synthetase (GCS) heavy

- subunit promoter. *Biochim Biophys Acta* 2001;1517:212-9.
15. Ohtsuji M, Katsuoka F, Kobayashi A, Aburatani H, Hayes JD, Yamamoto M. Nrf1 and Nrf2 play distinct roles in activation of antioxidant response element-dependent genes. *J Biol Chem* 2008;283:33554-62.
 16. Wang Y, Lou MF. The regulation of NADPH oxidase and its association with cell proliferation in human lens epithelial cells. *Invest Ophthalmol Vis Sci* 2009;50:2291-300.
 17. Marriott I. Osteoblast responses to bacterial pathogens: a previously unappreciated role for bone-forming cells in host defense and disease progression. *Immunol Res* 2004;30:291-308.
 18. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007;39:44-84.
 19. Chen L, Kwong M, Lu R, Ginzinger D, Lee C, Leung L, et al. Nrf1 is critical for redox balance and survival of liver cells during development. *Mol Cell Biol* 2003;23:4673-86.
 20. Durnam DM, Hoffman JS, Quaife CJ, Benditt EP, Chen HY, Brinster RL, et al. Induction of mouse metallothionein-I mRNA by bacterial endotoxin is independent of metals and glucocorticoid hormones. *Proc Natl Acad Sci U S A* 1984;81:1053-6.
 21. Durnam DM, Palmiter RD. Transcriptional regulation of the mouse metallothionein-I gene by heavy metals. *J Biol Chem* 1981;256:5712-6.
 22. Mayo KE, Palmiter RD. Glucocorticoid regulation of metallothionein-I mRNA synthesis in cultured mouse cells. *J Biol Chem* 1981;256:2621-4.
 23. Searle PF, Davison BL, Stuart GW, Wilkie TM, Norstedt G, Palmiter RD. Regulation, linkage, and sequence of mouse metallothionein I and II genes. *Mol Cell Biol* 1984;4:1221-30.
 24. Beach LR, Palmiter RD. Amplification of the metallothionein-I gene in cadmium-resistant mouse cells. *Proc Natl Acad Sci U S A* 1981;78:2110-4.
 25. Li DY, Xue MY, Geng ZR, Chen PY. The suppressive effects of Bursopentine (BP5) on oxidative stress and NF- κ B activation in lipopolysaccharide-activated murine peritoneal macrophages. *Cell Physiol Biochem* 2012;29:9-20.
 26. Hernandez-Montes E, Pollard SE, Vauzour D, Jofre-Montseny L, Rota C, Rimbach G, et al. Activation of glutathione peroxidase via Nrf1 mediates genistein's protection against oxidative endothelial cell injury. *Biochem Biophys Res Commun* 2006;346:851-9.
 27. Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y, et al. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. *J Biol Chem* 2000;275:16023-9.
 28. Moinova HR, Mulcahy RT. Up-regulation of the human gamma-glutamylcysteine synthetase regulatory subunit gene involves binding of Nrf-2 to an electrophile responsive element. *Biochem Biophys Res Commun* 1999;261:661-8.
 29. Kousteni S. FoxOs: unifying links between oxidative stress and skeletal homeostasis. *Curr Osteoporos Rep* 2011;9:60-6.
 30. Kim J, Xing W, Wergedal J, Chan JY, Mohan S. Targeted disruption of nuclear factor erythroid-derived 2-like 1 in osteoblasts reduces bone size and bone formation in mice. *Physiol Genomics* 2010;40:100-10.
 31. Xing W, Singgih A, Kapoor A, Alarcon CM, Baylink DJ, Mohan S. Nuclear factor-E2-related factor-1 mediates ascorbic acid induction of osterix expression via interaction with antioxidant-responsive element in bone cells. *J Biol Chem* 2007;282:22052-61.